Malaria transmission-blocking vaccines Pfs230D1-EPA and Pfs25-EPA in Alhydrogel in healthy Malian adults; a phase 1, randomised, controlled trial

Issaka Sagara, Sara A Healy*, Mahamadoun H Assadou, Mamady Kone, Bruce J Swihart, Jennifer L Kwan, Jonathan Fintzi, Kourane Sissoko, Bourama Kamate, Yacouba Samake, Merepen A Guindo, M'Bouye Doucoure, Karamoko Niaré, Amagana Dolo, Balla Diarra, Kelly M Rausch, David L Narum, David S Jones, Nicholas J MacDonald, Daming Zhu, J Patrick Gorres, Alemush Imeru, Rathy Mohan, Ismaila Thera, Irfan Zaidi, Fernando Salazar-Miralles, Junhui Duan, Jillian Neal, Robert D Morrison, Olga Muratova, Daman Sylla, Elise M O'Connell, Yimin Wu, Jen C C Hume, Mamadou B Coulibaly, Charles F Anderson, Sekou F Traore, Ogobara K Doumbo, Patrick E Duffy*

Summary

Background Malaria transmission-blocking vaccines target mosquito-stage parasites and will support elimination programmes. Gamete vaccine Pfs230D1-EPA/Alhydrogel induced superior activity to zygote vaccine Pfs25-EPA/Alhydrogel in malaria-naive US adults. Here, we compared these vaccines in malaria-experienced Malians.

Methods We did a pilot safety study then double-blind, block-randomised, comparator-controlled main-phase trial in malaria-intense Bancoumana, Mali. 18–50-year-old healthy non-pregnant, non-breastfeeding consenting adult residents were randomly assigned (1:1:1:1) to receive four doses at months 0, 1, 4·5, and 16·5 of either 47 µg Pfs25, 40 µg Pfs230D1 or comparator (Twinrix or Menactra)—all co-administered with normal saline for blinding—or 47 µg Pfs25 plus 40 µg Pfs230D1 co-administered. We documented safety and tolerability (primary endpoint in the as-treated populations) and immunogenicity (secondary endpoint in the as-treated populations: ELISA, standard-membrane-feeding assay, and mosquito direct skin feed assay). This trial is registered at ClinicalTrials.gov, NCT02334462.

Findings Between March 19, and June 2, 2015, we screened 471 individuals. Of 225 enrolled for the pilot and main cohorts, we randomly assigned 25 participants to pilot safety cohort groups of five (20%) to receive a two-dose series of Pfs25-EPA/Alhydrogel (16 µg), Pfs230D1-EPA/Alhydrogel (15 µg) or comparator, followed by Pfs25-EPA/Alhydrogel (16 µg) plus Pfs230D1-EPA/Alhydrogel (15 µg) or comparator plus saline. For the main cohort, we enrolled 200 participants between May 11 and June 2, 2015, to receive a four-dose series of 47 µg Pfs25-EPA/Alhydrogel plus saline (n=50 [25%]; Pfs25), 40 µg Pfs230D1-EPA/Alhydrogel plus saline (n=49 [25%]; Pfs230D1), 47 µg Pfs25-EPA/Alhydrogel plus 40 µg Pfs230D1-EPA/Alhydrogel (n=50 [25%]; Pfs25 plus Pfs230D1), or comparator (Twinrix or Menactra) plus saline (n=51 [25%]). Vaccinations were well tolerated in the pilot safety and main phases. Most vaccinees became seropositive after two Pfs230D1 or three Pfs25 doses; peak titres increased with each dose thereafter (Pfs230D1 geometric mean: 77·8 [95% CI 56·9–106·3], 146·4 [108·3–198·0], and 410·2 [301·6–558·0]; Pfs25 geometric mean 177·7 [130·3–242·4] and 315·7 [209·9–474·6]). Functional activity (mean peak transmission-reducing activity) appeared for Pfs230D1 (74·5% [66·6–82·5]) and Pfs25 plus Pfs230D1 (68·6% [57·3–79·8]), after the third dose and after the fourth dose (88·9% [81·7–96·2] for Pfs230D1 and 85·0% [78·4–91·5] Pfs25 plus Pfs230D1) but not for Pfs25 (58·2% [49·1–67·3] after the third dose and 58·2% [48·5–67·9] after the fourth dose). Pfs230D1 transmission-reducing activity (73·7% [64·1–83·3]) persisted 10 weeks after the fourth dose. Transmission-reducing activity of 80% was estimated at 1659 ELISA units for Pfs25, 218 for Pfs230D1, and 223 for Pfs230D1 plus Pfs25. After 3850 direct skin feed assays, 35 participants (12 Pfs25, eight Pfs230D1, five Pfs25 plus Pfs230D1, and ten comparator) had transmitted parasites at least once. The proportion of positive assays in vaccine groups (Pfs25 33 [3%] of 982 [–0·013 to 0·014], Pfs230D1 22 [2%] of 954 [–0·005 to 0·027], and combination 11 [1%] of 940 [–0·024 to 0·002]) did not differ from that of the comparator (22 [2%] of 974), nor did Pfs230D1 and combination groups differ (–0·024 to 0·001).

Interpretation Pfs230D1 but not Pfs25 vaccine induces durable serum functional activity in Malian adults. Direct skin feed assays detect parasite transmission to mosquitoes but increased event rates are needed to assess vaccine effectiveness.

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*Joint first authors

Malaria Research and Training Center, Mali- National Institute of Allergy and Infectious Diseases International Center for Excellence in Research, University of Sciences, Techniques and Technologies of Bamako, Mali (Prof I Sagara MD, M H Assadou MD, M Kone MD, K Sissoko MD, B Kamate MD, Y Samake MD, M A Guindo PharmD, M Doucoure MSc, K Niaré PharmD, Prof A Dolo PharmD, B Diarra PharmD, D Sylla MD, I Thera MSc, M B Coulibaly PharmD PhD, Prof S F Traore PhD, Prof O K Doumbo MD); **Laboratory of Malaria Immunology and Vaccinology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA** (S A Healy MD, K M Rausch MS, D L Narum PhD, D L Jones PhD, N J MacDonald PhD, D Zhu MS, J P Gorres MPH, A Imeru MPH, R Mohan MS, I Zaidi PhD, F Salazar-Miralles BS, J Duan PhD, J Neal BA, R D Morrison MS, O Muratova MS, Y Wu PhD, J C C Hume DPhil, C Anderson PhD, P E Duffy MD); **Biostatistics Research Branch** (B Swihart PhD, J Fintzi PhD), **Laboratory of Clinical Immunology and Microbiology** (J Kwan PhD) **Laboratory of Parasitic Diseases** (E O'Connell MD), **National**

Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Correspondence to:
Dr Patrick E Duffy, Laboratory of Malaria Immunology and Vaccinology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-8180, USA
patrick.duffy@nih.gov

Research in context

Evidence before this study

We searched PubMed, the Cochrane Library, and other relevant data sources for randomised trials of malaria vaccines in adults published in English between Jan 1, 1980, and Feb 27, 2023. We searched using the following terms ("malaria vaccines" [MeSH Terms] OR "malaria"[All Fields] AND "vaccines"[All Fields]) OR "malaria vaccines"[All Fields] OR ("malaria"[All Fields] AND "vaccine"[All Fields]) OR "malaria vaccine"[All Fields]) AND (Pfs25 [All Fields] AND Pfs230 [All Fields]))). For the Cochrane Library and other data sources, we used the key search terms "Pfs25", "Pfs230", "malaria vaccines", AND "clinical trials". Although transmission-blocking vaccine studies have been previously done in malaria-endemic regions, no trial of a Pfs230 vaccine in malaria-experienced populations has been published. Another leading transmission-blocking vaccine target is gamete surface protein Pfs48/45, a long-established prime candidate that induces antibodies that prevent parasite development in the mosquito vector. Pfs48/45 first entered clinical trials in 2021 in the Netherlands in the form of subunit R0.6C, after overcoming nearly 20 years of challenges to manufacturing, particularly yielding sufficient quantities of a properly folded, functional activity-inducing protein. We previously reported that Pfs25 or Pfs230 domain 1 vaccines (prepared as *Pichia*-expressed recombinant proteins conjugated to carrier-protein ExoProtein A [EPA] and formulated in the alum-based adjuvant Alhydrogel: Pfs25H-EPA/Alhydrogel and Pfs230D1-EPA/Alhydrogel) were well tolerated and induced functional antisera in US volunteers. Pfs25H-EPA/Alhydrogel also induced functional antisera in malaria-experienced Malian volunteers, and reduced *Plasmodium falciparum* transmission to mosquitoes in a laboratory assay, but Pfs25 titres decayed rapidly after peak, and functional activity seen at peak titres in US vaccinees was lost by 8 weeks after vaccination. In US volunteers, the Pfs230D1 vaccine induced serum functional activity superior to that of Pfs25 after two doses, and the combination of Pfs230D1 plus Pfs25 did not increase serum activity.

Added value of this study

Here, we compared Pfs230D1 and Pfs25 vaccines, alone or in combination, as four-dose regimens in a malaria-experienced target population in Mali. Both vaccines were safe, well tolerated and, immunogenic in Malian adults. As seen in the US trial, Pfs230D1 was superior to Pfs25 for functional immunogenicity by standard membrane feeding assay; combining Pfs230 with Pfs25 did not improve serum functional activity.

Implications of all the available evidence

Pfs230D1-EPA is now the leading malaria transmission-blocking vaccine candidate. Both Pfs230D1-EPA and Pfs25-EPA candidates are safe and can be assessed with more potent adjuvants to enhance functional antibody responses. The direct skin feeding assay measures wild-type parasite transmission to mosquitoes but will require increased event rates to establish vaccine effectiveness in future field trials.

Introduction

Malaria is a global scourge with a marked increase in cases and deaths since 2019, mostly due to *Plasmodium falciparum*.[2] Existing tools are insufficient to achieve global eradication or even elimination in many countries where malaria remains entrenched. New interventions are urgently needed.

In 2021, WHO recommended[3] wider use of the first approved anti-parasite vaccine RTS,S/AS01 (GSK Vaccines, Rixensart, Belgium), which reduces clinical malaria and malaria deaths in individuals younger than 5 years. Candidates R21/Matrix M[4] (Serum Institute of India Private Limited, Pune, India) and PfSPZ Vaccine[5] (Sanaria, Rockville, MD, USA) have shown efficacy in field trials, and similar to RTS,S/AS01, target pre-erythrocytic parasites (sporozoite and liver stages).

Malaria transmission-blocking vaccines target gametes or zygotes in the mosquito to block transmission[6,7] and can be used for elimination. Transmission-blocking vaccines have no activity against pre-erythrocytic or blood-stage parasites.[6,7] Leading transmission-blocking vaccine candidate antigens include gamete surface proteins Pfs230 and Pfs48/45 and zygote and ookinete surface protein Pfs25.[8,9] We previously reported that *Pichia*-expressed Pfs25 conjugated to carrier-protein ExoProtein A (EPA) and formulated in Alhydrogel (Pfs25H-EPA/Alhydrogel) was well-tolerated and induced functional antisera in US[10] and Malian volunteers[1] and reduced *P falciparum* transmission to mosquitoes in a laboratory assay. However, Pfs25 titres decayed rapidly and functional activity measured 2 weeks after the fourth dose was lost by 8 weeks.[1,10] We subsequently reported *Pichia*-expressed Pfs230 domain 1 (Pfs230D1) vaccine (Pfs230D1-EPA/Alhydrogel) induced serum functional activity superior to Pfs25-EPA/Alhydrogel after two vaccine doses in malaria-naive US volunteers.[11]

Vaccine responses can be impaired in malaria-exposed populations.[1] Here, we compared Pfs230D1-EPA/Alhydrogel and Pfs25-EPA/Alhydrogel, alone or in combination, in a malaria-experienced target population in Mali.

Methods

Study design and participants

We did a phase 1 randomised controlled trial at John LaMontagne Malaria Research Center in Bancoumana, Mali, approximately 60 km southwest of Bamako, with highly seasonal (June to December) hyperendemic malaria transmission. Cohort enrollment was staggered for safety; participants in the Pfs25 plus Pfs230D1 group were enrolled after single-antigen (Pfs25 or

Pfs230D1) vaccine immunisations were reviewed for safety (appendix pp 6–7, 40). Participants in the main cohort received dosing schedules of 0, 1, 4·5, and 16·5 months (rather than planned 0, 1, 6, and 18 months) to complete vaccination before malaria transmission seasons; follow up ended approximately 6 months after the fourth dose.

Healthy study village residents aged 18–50-years (men or non-pregnant, non-breastfeeding women; residency established in village census) were eligible to enroll after informed consent, if available for trial duration and (for the main cohort) willing to undergo direct skin feed (DSF) mosquito assays. Women of child-bearing potential used reliable contraception during vaccinations. Individuals were excluded for abnormal laboratory results (including HIV or hepatitis B or C tests), previous malaria vaccine, or licensed vaccines (within 4 weeks [live] or 2 weeks [killed]), blood products, or immunosuppressive drugs. Full inclusion and exclusion criteria are given in the appendix (pp 9–11).

The trial adhered to Good Clinical Practice and institutional procedures and guidelines. Participating villages provided community permission; participants provided informed consent.[12] The study was approved in Mali (Faculté de Médecine de Pharmacie et d'OdontoStomatologie ethics committee and Mali national regulatory authority) and the USA (National Institute of Allergy and Infectious Diseases [NIAID] institutional review board), registered at ClinicalTrials.gov (NCT02334462), and conducted under the US Food and Drug Administration investigational new drug number 16251. Safety was monitored by an independent data and safety monitoring board and local medical monitor.

Randomisation and masking

We randomly assigned participants at enrollment to receive (1) Pfs25-EPA/Alhydrogel (pilot 16 µg Pfs25; main 47 µg Pfs25 plus saline), (2) Pfs230D1-EPA/Alhydrogel (pilot 15 µg Pfs230D1; main 40 µg Pfs230D1 plus saline); (3) combination (pilot 16 µg Pfs25 plus 15 µg Pfs230D1; main 47 µg Pfs25 plus 40 µg Pfs230D1); or (4) comparator (Twinrix [GSK Vaccines, Rixensart, Belgium] for vaccinations 1, 2, and 3, and Menactra [Sanofi Pasteur, Swiftwater, PA, USA] for the fourth) with or without saline as needed for blinding. The study investigators, vaccinators, laboratory personnel, and participants were masked to treatment group assignment. Unmasked site pharmacists received randomisation codes via secure email. Product syringes were masked by opaque tape and labeled only with the study identification number. Group assignments were unmasked at final study visit: approximately 6 months after the second dose in the pilot safety cohort and approximately 6 months after the fourth dose in the main cohort. After unmasking, investigational vaccine groups were offered Twinrix and Menactra.

Vaccines

See **Online** for appendix

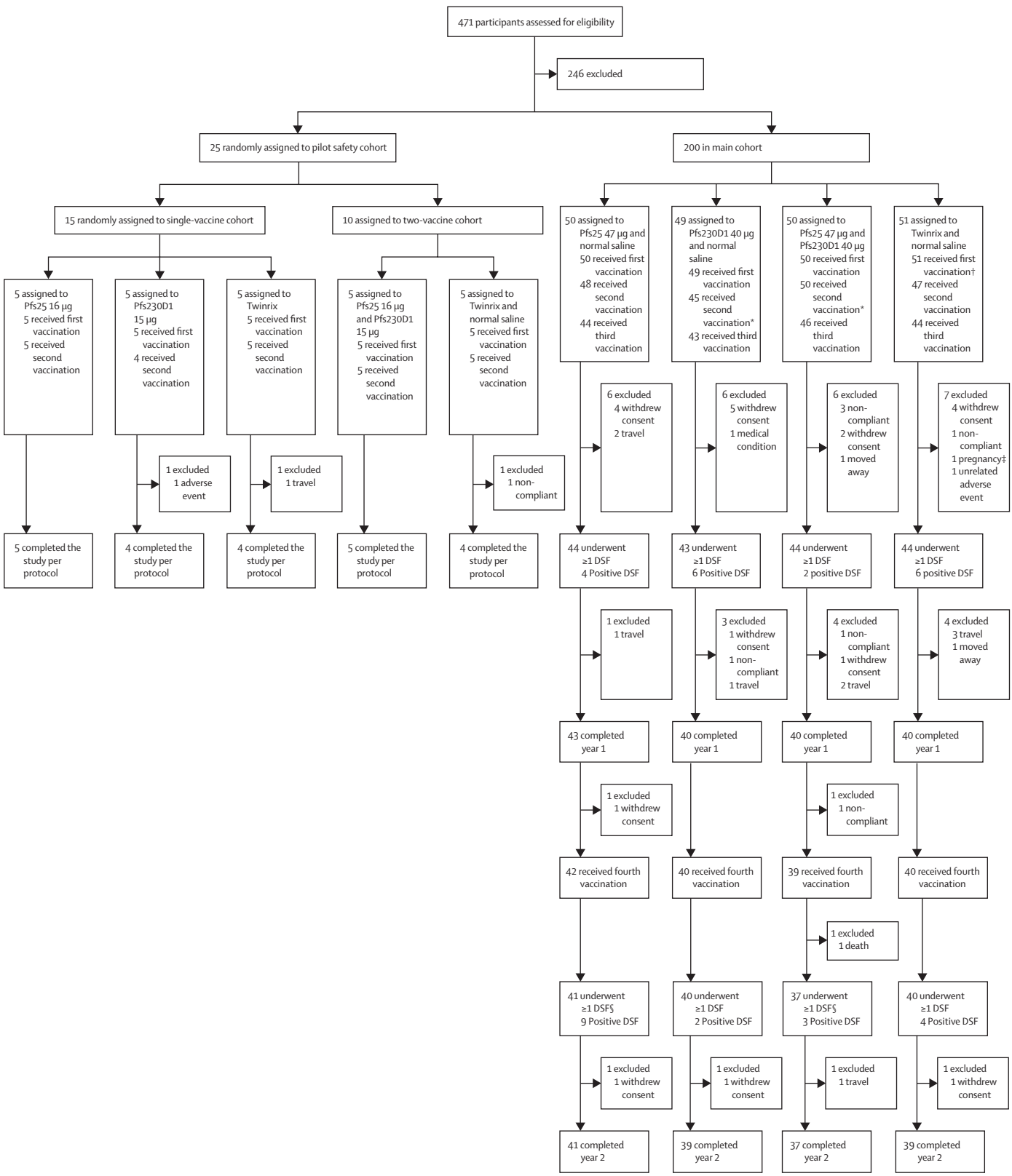
Pfs25-EPA/Alhydrogel and Pfs230D1-EPA/Alhydrogel vaccines consist of *Pichia pastoris*-expressed recombinant Pfs25[13] (UniProt 25 kDa ookinete surface antigen) and Pfs230D1 (UniProt gametocyte surface protein P230),[14] respectively (manufactured at the Walter Reed Army Institute of Research Pilot Bioproduction Facility [WRAIR PBF], Silver Spring, MD, USA) conjugated to *Escherichia coli*-expressed recombinant *Pseudomonas aeruginosa* EPA[15] (WRAIR PBF) and adjuvanted with Alhydrogel (Brenntag, Frederikssund, Denmark). Additional vaccine information is in the appendix (pp 7–9).

Procedures

Per study design (appendix p 40), pilot safety cohorts (n=25) received two doses at months 0 and 1 of either 0·2 mL (Pfs25, 16 µg) or 0·3 mL (Pfs230D1, 15 µg), or both, or comparator vaccine Twinrix (1 mL recombinant hepatitis A and B vaccine) with (n=5) or without (n=5) 1 mL saline (0·9% sodium chloride injection [USP, Hospira, Lake Forest, IL, USA]). Participants in the main cohort (n=200) received 0·6 mL (Pfs25, 47 µg) or 0·8 mL (Pfs230D1, 40 µg), or both, or comparator at months 0, 1, 4·5, and 16·5; single antigen (Pfs25, Pfs230D1, or comparator) was co-administered with 1 mL saline to maintain masking. Participants in the comparator group received Twinrix at months 0, 1, and 4·5 in year 1, and Menactra (0·5 mL meningococcal polysaccharide vaccine) at month 16·5. Local pediatricians not involved in follow-up or adverse event (AE) assessment completed deltoid injections in alternating (pilot safety cohort only) or both arms (co-administration, alternating with successive doses in both cohorts). Participants were considered enrolled upon first vaccination.

Participants were monitored 30 min after vaccination for AEs, then on days 1, 3, 7, 14, and 28, then monthly until unmasking. Medical personnel were always available for unscheduled visits. Solicited AEs were recorded 14 days after each vaccination (appendix p 41). Unsolicited AEs, including symptomatic malaria, serious AEs (SAEs), and new onset of chronic illnesses were recorded throughout the study. SAEs include death, a life-threatening event, inpatient hospitalisation, persistent or significant incapacity, a congenital anomaly or birth defect, or medically important event. Complete blood counts with differential, creatinine, alanine aminotransferase, and urinalysis were assessed before vaccination and 3 and 14 days after each vaccination. We used US Food and Drug Administration guidelines[16] for AE grading, adapted to local laboratory reference ranges (appendix pp 42–43).

Blood smears were prepared before vaccination and at least monthly after vaccination, twice per week with DSF visits following the third and fourth vaccinations, and when clinically indicated. Symptomatic malaria was defined as asexual parasitemia with axillary temperature at least 37·5°C or clinical signs or symptoms of malaria,


471 participants assessed for eligibility
246 excluded
25 randomly assigned to pilot safety cohort
200 in main cohort
15 randomly assigned to single-vaccine cohort
10 assigned to two-vaccine cohort
5 assigned to Pfs25 16 µg
5 received first vaccination
5 received second vaccination
5 assigned to Pfs230D1 15 µg
5 received first vaccination
4 received second vaccination
5 assigned to Twinrix
5 received first vaccination
5 received second vaccination
5 assigned to Pfs25 16 µg and Pfs230D1 15 µg
5 received first vaccination
5 received second vaccination
5 assigned to Twinrix and normal saline
5 received first vaccination
5 received second vaccination
1 excluded
1 adverse event
1 excluded
1 travel
1 excluded
1 non-compliant
5 completed the study per protocol
4 completed the study per protocol
4 completed the study per protocol
5 completed the study per protocol
4 completed the study per protocol
50 assigned to Pfs25 47 µg and normal saline
50 received first vaccination
48 received second vaccination
44 received third vaccination
49 assigned to Pfs230D1 40 µg and normal saline
49 received first vaccination
45 received second vaccination*
43 received third vaccination
50 assigned to Pfs25 47 µg and Pfs230D1 40 µg
50 received first vaccination
50 received second vaccination*
46 received third vaccination
51 assigned to Twinrix and normal saline
51 received first vaccination†
47 received second vaccination
44 received third vaccination
6 excluded
4 withdrew consent
2 travel
6 excluded
5 withdrew consent
1 medical condition
6 excluded
3 non-compliant
2 withdrew consent
1 moved away
7 excluded
4 withdrew consent
1 non-compliant
1 pregnancy‡
1 unrelated adverse event
44 underwent ≥1 DSF
4 Positive DSF
43 underwent ≥1 DSF
6 Positive DSF
44 underwent ≥1 DSF
2 positive DSF
44 underwent ≥1 DSF
6 positive DSF
1 excluded
1 travel
3 excluded
1 withdrew consent
1 non-compliant
1 travel
4 excluded
1 non-compliant
1 withdrew consent
2 travel
4 excluded
3 travel
1 moved away
43 completed year 1
40 completed year 1
40 completed year 1
40 completed year 1
1 excluded
1 withdrew consent
1 excluded
1 non-compliant
42 received fourth vaccination
40 received fourth vaccination
39 received fourth vaccination
40 received fourth vaccination
1 excluded
1 death
41 underwent ≥1 DSF§
9 Positive DSF
40 underwent ≥1 DSF
2 Positive DSF
37 underwent ≥1 DSF§
3 Positive DSF
40 underwent ≥1 DSF
4 Positive DSF
1 excluded
1 withdrew consent
1 excluded
1 withdrew consent
1 excluded
1 travel
1 excluded
1 withdrew consent
41 completed year 2
39 completed year 2
37 completed year 2
39 completed year 2

and was treated with antimalarials; asymptomatic parasitemia was not treated as per Malian Government guidelines. Blood smears were examined under standard procedures by trained technicians with skills regularly documented using masked blood smear sets.

Immunogenicity samples were collected at various timepoints (appendix pp 44–45). Antibodies to Pfs25, Pfs230D1, and EPA were measured by ELISA on vaccination day, 14 days after vaccination, then periodically after the third and fourth vaccinations, using previously published methods.[1,10,11] For descriptive analyses of raw data, seropositive ELISA values were defined as greater than the mean limit of detection, on the basis of the standard curve for each plate averaged across all ELISA plates. Supplementary modeling of ELISA data used plate-specific limit of detection.

Serum functional activity was measured by standard membrane feeding assay (SMFA) in which mosquitoes feed on cultured gametocytes in the presence of test (immune) or control (naive) sera, using previously published methods.[10] At least 20 mosquitoes were dissected approximately 1 week after feeding to count infected mosquitoes and parasites (oocysts) per infected mosquito. Transmission-reducing-activity was defined as ((mean-oocyst-count in control sera – mean oocyst count in test sera)/mean-oocyst-count in control sera) × 100. Transmission-blocking-activity was defined as (mean-infection prevalence in control sera – mean-infection prevalence in test sera) / mean-infection prevalence in control sera) × 100.

To assess vaccine effectiveness, DSF assays were done twice per week for 6 weeks in all main cohort participants (regardless of blood smear results) starting 7 days after the third or fourth vaccinations for a maximum of 12 DSFs each year. In brief (details in appendix pp 16–18), two mesh-covered feeding pints, each containing up to 30 (first year) or 15 (second year) pre-starved laboratory-adapted female *Anopheles coluzzi* were placed on participants' calfs or forearms by trained entomology staff to blood-feed for 15-20 min. Participants were then offered topical antihistamine or topical antipruritic and followed up for AEs. Only fed mosquitoes were transported to Bamako, stored in the secure insectary, and dissected a week later to count oocysts. Midguts of a subset of infected mosquitoes were PCR tested for parasite speciation (appendix pp 19–21).

Experimental hut studies (appendix pp 18–19) were explored to measure transmission and vaccine efficacy, whereby blood smear-positive individuals slept alone in huts modified to limit mosquito ingress and egress overnight; bloodfed mosquitoes that were captured in the morning underwent forensic testing of human blood source.

We assessed schistosomiasis and stool helminth and protozoan infections at baseline in the main cohort using previously published methods (appendix p 19).

Figure 1: **Trial profile**
Trial included pilot safety cohort then main cohort. For the pilot safety cohort, "completed study per protocol" was defined as completed until study day 196 (approximately 6 months after second dose). For the main cohort, "completed year 1" was defined as having completed until study day 510 (approximately 11 months after third dose; end of year 1) and "completed year 2" was defined as having completed until study day 730 (approximately 6 months poafter fourth dose). DSF year 1 was defined as completing at least one DSF assay from study day 175 (7 days after third dose) to study day 213 (45 days after third dose). DSF year 2 was defined as completing at least one DSF assay from study day 547 (7 days after fourth dose) to study day 585 (45 days after fourth dose). DSF=direct skin feed. ITT=intention to treat. *One participant randomly assigned to Pfs230D1 was administered 47 µg Pfs25 plus 40 µg Pfs230D1 for the second vaccination and included in the Pfs230D1 group for both as-treated and ITT analyses. †One participant randomly assigned to 40 µg Pfs230D1 plus normal saline was erroneously administered comparator for the first vaccination, then continued to receive comparator throughout the study (participant and clinical team remained masked) and for the analysis was included in the comparator group (for as-treated analysis) the Pfs230D1 group (for ITT analysis). ‡One participant (Twinrix plus normal saline) became pregnant just before the second vaccination and was intentionally unmasked early for counseling of risk given vaccine received. §Two participants (one Pfs25 and one Pfs25 plus Pfs230D1) did not complete a single DSF in year 2 but completed the study.

Outcomes

The primary outcome (as-treated population) in the pilot safety and main cohorts was safety, tolerability, and reactogenicity of repeated immunisation with increasing doses of vaccines, based on the occurrence and severity of local AEs, systemic AEs, and laboratory abnormalities after each vaccination. Unsolicited AEs (including symptomatic malaria), SAEs, unanticipated problems, and new onset of chronic illness were reported throughout the study. AEs related to DSF were recorded for 7 days after feed.

The secondary outcomes (as-treated population) in the main cohort were immunogenicity, measured starting 2 weeks after vaccination as seroreactivity by IgG ELISA, antibody functionality by SMFA (2 weeks after the third dose, and 2 and [for Pfs230D1 and comparator groups] 10 weeks after the fourth dose), and vaccine effectiveness against parasite transmission to mosquitoes, measured by DSF done across two malaria seasons. Additional analyses to assess seropositive rates, antibody durability, ELISA relationships to SMFA and DSF, and similarity between Malian and US vaccinee responses were done on an exploratory basis. Similar analyses (ELISA and SMFA) were completed in the pilot safety cohort as exploratory objectives. Additional exploratory analyses were completed and are reported in the appendix (pp 12–13, 18–23).

Statistical analysis

Primary safety endpoint was analysed by as-treated and intention to treat (ITT), which differed by a single participant (figure 1); thus, only the as-treated analysis is reported. Secondary immunogenicity endpoints (ELISA, SMFA, and DSF) were analysed by as-treated. The two pilot comparator groups were combined for analyses. Safety analyses included all participants who received at

least one vaccine dose and examined AEs as proportions of unique participants (Fisher's exact and χ^2 tests) and overall counts (Wilcoxon-Mann-Whitney tests), including by grade and relationship to vaccination. Descriptive statistics for proportions were assessed by modified Wald test with 95% CIs. Categorical data were analysed by two-tailed Fisher's Exact or χ^2 tests.

We compared ELISA levels between groups by Wilcoxon-Mann-Whitney test at specific timepoints. We compared seroconversion rates using conditional exact test for given timepoints (R package exact2x2). As supplementary analysis, we fit two Bayesian proportional odds models for the number of doses required to elicit an immune response. Antibody decay profiles were also modeled with a hierarchical Bayesian model. Modeling details are provided in the appendix (pp 21–23).

Group SMFA measures were compared using Kruskal-Wallis with Dunn's correction for multiple comparisons, whereas individual comparison between Pfs230D1 alone versus comparator 10 weeks after the fourth dose were

	Pilot safety cohort				Main cohort			
	Pfs25 16 µg (n=5)	Pfs230D1 15 µg (n=5)	Pfs25 16 µg plus Pfs230D1 15 µg (n=5)	TWINRIX with or without normal saline (n=10)	Pfs25 47 µg plus normal saline (n=50)	Pfs230D1 40 µg plus normal saline (n=49)*	Pfs25 47 µg plus Pfs230D1 40 µg (n=50)	TWINRIX or Menactra plus normal saline (n=51)*
Sex								
Male	4 (80%)	4 (80%)	4 (80%)	8 (80%)	35 (70%)	33 (67%)	36 (72%)	36 (71%)
Female	1 (20%)	1 (20%)	1 (20%)	2 (20%)	15 (30%)	16 (33%)	14 (28%)	15 (29%)
Age, years								
Mean (SD)	35·6 (12·5)	38·6 (8·9)	34·6 (10·3)	31·5 (10·2)	38 (9·3)	38·3 (8·8)	36·6 (9·5)	37·8 (10·4)
Range	19–50	29–48	19–46	19–47	18–50	19–50	19–50	18–50
Village								
Bancoumana	5 (100%)	5 (100%)	5 (100%)	10 (100%)	29 (58%)	24 (49%)	28 (56%)	22 (43%)
Koursale	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (10%)	6 (12%)	7 (14 %)	7 (14%)
Kolle	0 (0%)	0 (0%)	0 (0%)	0 (0%)	6 (12%)	6 (12%)	3 (6 %)	3 (6%)
Samako	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (10%)	4 (8%)	6 (12 %)	8 (16%)
Nankilabougou	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)
Missira	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	1 (2%)	0 (0%)
Djiguidala	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)	1 (2%)	1 (2%)	2 (4%)
Tema	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)	2 (4%)	1 (2%)	3 (6%)
Djoliba	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (4%)	6 (12%)	2 (4%)	6 (12%)
Co-infections†								
Urinary *Schistosoma haematobium*	0 (0%)	0 (0%)	0 (0%)	1 (10%)	3 (6%)	2 (4%)	4 (8%)	5 (10%)
Stool helminth	1 (25%)	0 (0%)	1 (20%)	2 (20%)	7 (23%)	4 (15%)	7 (21%)	3 (12%)
Stool protozoa	2 (50%)	2 (50%)	0 (0%)	5 (50%)	14 (45%)	15 (56%)	13 (39%)	6 (23%)
Stool (no result)‡	1 (20%)	1 (20%)	0 (0%)	0 (0%)	19 (38%)	22 (45%)	17 (34%)	25 (49%)
Parasitemia§								
Before first dose	1 (20%)	1 (20%)	1 (20%)	0 (0%)	4 (8%)	6 (12%)	6 (12%)	9 (18%)
Before fourth dose¶	NA	NA	NA	NA	4 (10%)	7 (18%)	0 (0%)	6 (15%)
Gametocytemia§								
Before first dose	1 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (4%)	1 (2%)	4 (8%)
Before fourth dose¶	NA	NA	NA	NA	5 (12%)	8 (20%)	2 (5%)	1 (3%)

Data are n (%), unless otherwise indicated. Age presented is age at enrollment (day of first vaccine). Pfs25=Pfs25-EPA/Alhydrogel. Pfs230=Pfs230D1-EPA/Alhydrogel. NA =not applicable. *One participant randomly assigned to Pfs230D1 40 µg plus normal saline was erroneously administered comparator for the first vaccination, which was reviewed by the study team, statistician, sponsor, and data and safety monitoring board who recommended the participant continue to receive comparator for the rest of the study (participant and clinical team remained masked). For the analysis this participant was considered to be in the comparator group (as-treated analysis) and Pfs230D1 group (intention-to-treat analysis). †Urine and stool samples were obtained from participants during screening. Urine schistosomiasis screening was completed on site and positive participants were treated with praziquantel; all enrolled participants underwent urinary schistosomiasis testing. Urinary *Schistosoma haematobium* was further quantified for density of infection (slightly infected 1–49 eggs per 10 mL of urine, moderately infected 50–100 eggs per 10 mL, and heavily infected ≥100 eggs per 10 mL of urine)—all reported infections were slightly infected except one participant with a heavily infected sample (main cohort TWINRIX or Menactra plus normal saline). Stool PCR was completed retrospectively at the US National Institutes of Health and did not affect clinical care. Testing was completed for the following pathogens and grouped accordingly: helminth: *Ascaris lumbricoides* (nematode or roundworm), *Trichuris trichiura* (nematode or roundworm), *Strongyloides stercoralis* (nematode or roundworm), *Necator americanus* (hookworm), *Ancylostoma duodenale* (hookworm); protozoa: *Giardia lamblia, Cryptospordium parvum/hominis,* and *Entamoeba histolytica*. ‡Stool not collected or assay not completed, or negative internal control, therefore we cannot rule out false negatives due to PCR inhibition. Calculated percentages for stool results are based on assayed results (in the pilot safety cohort Pfs25 n=4, Pfs230 n=4, Pfs25 plus Pfs230 n=5, and Twinrix with or without normal saline n=10; and in the main cohort Pfs25 plus normal saline n=31, Pfs230D1 plus normal saline n=27, Pfs25 plus Pfs230 n=33, and Twinrix or Menactra plus normal saline n=26). §Parasitemia was defined as blood smear greater than 0 asexual *Plasmodium falciparum* on day of first vaccine (study day 0) or on day of fourth vaccine (main cohort only; study day 540). Gametocytemia was defined as blood smear greater than 0 gametocytes (sexual *P falciparum*) seen by at least one reader on day of first vaccine (study day 0) or on day of fourth vaccine (main cohort only; study day 540). ¶For before the fourth dose, n per group (main cohort only) became n=42 in Pfs25 plus normal saline, n=40 in Pfs230D1 plus normal saline, n=39 in Pfs25 plus Pfs230, and n=40 in Twinrix or Menactra plus normal saline.

Table 1: **Baseline characteristics**

	Pfs25 47 µg plus normal saline				Pfs230 40 µg plus normal saline				Pfs25 47 µg plus Pfs230 40 µg				Twinrix or Menactra plus normal saline			
	First dose (n=50)	Second dose (n=48)	Third dose (n=44)	Fourth dose (n=42)	First dose (n=49)	Second dose (n=45)	Third dose (n=43)	Fourth dose (n=40)	First dose (n=50)	Second dose (n=50)	Third dose (n=46)	Fourth dose (n=39)	First dose (n=51)	Second dose (n=47)	Third dose (n=44)	Fourth dose (n=40)
Total adverse events	37* (74%); 70	45 (94%); 133	41 (93%); 249	40 (95%); 179	35 (71%); 67	40 (89%); 105	39 (91%); 189	39 (98%); 152	36 (72%); 82	43 (86%); 126	44 (96%); 282	37 (95%); 178	27 (53%); 48	42 (89%); 103	43 (98%); 225	40 (100%); 149
Grade 1	33* (66%); 49	38 (79%); 67	39 (89%); 85	20 (48%); 26	28* (57%); 46	36 (80%); 57	29 (67%); 68	20 (50%); 33	32* (64%); 60	38 (76%); 88	38 (83%); 103	21 (54%); 36	18 (35%); 29	36 (77%); 62	32 (73%); 75	18 (45%); 24
Grade 2	16 (32%); 21	34 (71%); 65	40 (91%); 156	39 (93%); 151	17 (35%); 21	29 (64%); 46	32 (74%); 115	37 (93%); 117	17 (34%); 21	26 (52%); 37	42 (91%); 176	34 (87%); 139	16 (31%); 18	26 (55%); 38	43 (98%); 145	40 (100%); 124
Grade 3	0	2 (4%); 2	8 (18%); 8	2 (5%); 2	0	2 (4%); 2	4 (9%); 5	2 (5%); 2	1 (2%); 1	1 (2%); 1	3 (7%); 3	2 (5%); 2	0	2 (4%); 3	4 (9%); 5	1 (3%); 1
Grade 4	0	0	0	0	0	0	1 (2%); 1	0	0	0	0	0	1 (2%); 1	0	0	0
Grade 5	0	0	0	0	0	0	0	0	0	0	0	1 (3%); 1	0	0	0	0
Related adverse events	31* (62%); 44	35* (73%); 52	20* (46%); 28	21 (50%); 25	23 (47%); 33	28* (62%); 31	18* (42%); 23	17 (43%); 20	29* (58%); 53	26 (52%); 48	23* (50%); 45	23* (59%); 37	16 (31%); 19	17 (36%); 23	10 (23%); 12	12 (30%); 17
Serious adverse events	0	0	0	0	0	1 (2%); 1	1 (2%); 1	0	0	0	0	1 (3%); 1	0	0	0	0
Malaria adverse events†	2 (4%); 2	13 (27%); 13	29 (66%); 37	25 (60%); 32	2 (4%); 2	12 (27%); 12	24 (56%); 29	20 (50%); 25	0	12 (24%); 13	29 (63%); 40	22 (56%); 26	2 (4%); 2	16 (34%); 17	31 (71%); 44	22 (55%); 26
Local reactogenicity	26* (52%); 32	31* (65%); 42	16* (36%); 23	20 (48%); 23	21* (43%); 24	22 (49%); 22	17* (40%); 21	16 (40%); 16	26* (52%); 48	23 (46%); 43	22* (48%); 40	17 (44%); 28	6 (12%); 9	14 (30%); 16	4 (9%); 4	10 (25%); 15
Solicited reactogenicity	3 (6%); 3	3 (6%); 3	4 (9%); 4	3 (7%); 4	5 (10%); 5	3 (7%); 3	1 (2%); 1	5 (13%); 5	2 (4%); 2	3 (6%); 3	2 (4%); 3	8* (21%); 8	5 (10%); 5	4 (9%); 4	2 (5%); 3	1 (3%); 1
Laboratory adverse event	8 (16%); 8	8 (17%); 9	4 (9%); 4	0	5 (12%); 5	8 (18%); 10	2 (5%); 2	5 (13%); 5	4 (8%); 4	5 (10%); 5	2 (4%); 2	5 (13%); 6	7 (14%); 8	6 (13%); 7	6 (14%); 7	4 (10%); 4

Data are number of unique participants experiencing adverse events (percentage of participants with adverse events); absolute number of adverse events. Reporting periods for adverse events were protocol specific. Unsolicited adverse events, serious adverse events, unanticipated problems, and new-onset chronic illnesses were recorded until the end of the study. The following reporting periods were defined: during entire study period (for first vaccination approximately 1 month, second vaccination 3·5 months, third vaccination 12 months, and fourth vaccination 6 months); local reactogenicity was assessed until 7 days after vaccination; solicited reactogenicity was assessed until 14 days after vaccination; laboratory adverse events were assessed until 14 days after vaccination plus visit window timeframe (+3 days). Local injection site reactogenicity included pain or tenderness, erythema or redness, swelling, induration, and pruritus. Systemic solicited reactogenicity included fever, headache, nausea, malaise, myalgia, arthralgia, and urticaria. Scheduled laboratory tests (complete blood cell count with differential, alanine transaminase, and creatinine) were completed 3 and 14 days after vaccination. Given all participants received two vaccinations (co-administration), if local reactogenicity was reported and attributed to both upper arms, two individual adverse events were reported in one participant. Symptomatic malaria was reported as an adverse event (defined as *Plasmodium* asexual parasitaemia accompanied by an axillary temperature of at least 37·5°C or clinical signs and symptoms compatible with malaria) and collected throughout the study duration. All adverse events were coded using MedDRA and preferred terms provided. *Differs significantly from the control. †For fair comparison between study year 1 (2015) and year 2 (2016), symptomatic malaria cases reported were assessed for a 6-month period after the third and fourth doses

Table 2: **Safety summary of main cohort**

analysed using Wilcoxon-Mann-Whitney. The association of SMFA with longitudinal ELISA values was assessed using generalised estimating equations and estimates for ELISA values that would achieve 80% transmission-reducing activity by linear regression. We assessed DSF oocyst counts in zero-inflated negative binomial random effect models, with the traditional log-link and normally distributed random effects (this model has a parameter to account for excess 0 counts and the negative binomial accounts for overdispersion better than Poisson models).

Role of the funding source

NIAID scientists but not NIAID officials were involved in study design, study management, data collection, analysis, interpretation, report writing, and decision to submit.

Results

Between March 19, and June 2, 2015, we screened 471 individuals. Of 225 enrolled for the pilot and main cohorts, we randomly assigned 25 to pilot safety cohort groups of five to receive a two-dose series of Pfs25-EPA/Alhydrogel (16 µg), Pfs230D1-EPA/Alhydrogel (15 µg) or comparator, followed by Pfs25-EPA/Alhydrogel (16 µg) plus Pfs230D1-EPA/Alhydrogel (15 µg) or comparator plus saline (figure 1). First vaccinations occurred in April 2015, and the last vaccinations in May 2015, and scheduled unmasking occurred in November 2015 (figure 1; appendix p 40). Nine (90%) of ten individuals who received comparator and 13 (87%) of 15 who received experimental vaccines continued to the pilot safety study end.

For the main cohort, we enrolled 200 participants between May 11 and June 2, 2015, to receive four-dose series of 47 µg Pfs25-EPA/Alhydrogel plus saline (n=50 [25%]; Pfs25), 40 µg Pfs230D1-EPA/Alhydrogel plus saline (n=49 [25%]; Pfs230D1), 47 µg Pfs25-EPA/Alhydrogel plus 40 µg Pfs230D1-EPA/Alhydrogel (n=50 [25%]; Pfs25 plus Pfs230D1), or comparator (Twinrix or Menactra) plus saline (n=51 [25%]; figure 1; appendix p 40). One participant randomly assigned to Pfs230D1 erroneously received the comparator at first vaccination; this participant was not unmasked and continued receiving the comparator. Vaccine doses in 2015 were given from May 11 to June 2, June 9–25, Sept 15 to Oct 16, and booster dose in 2016 from Sept 15 to Oct 17 (figure 1; appendix p 40). DSFs were done after the third dose (Sept 22 to Dec 2, 2015) and after the fourth dose (Sept 22 to Nov 30, 2016). All randomly assigned participants received at least one vaccination and were eligible for safety analyses (as-treated). Dropout was similar across groups, with 78% (range 74–82) completing the 2-year study including DSF (figure 1).

Study groups in the main cohort were similar, with mostly male participants (140 [70%] of 200 [95% CI 63·3–76·0]) drawn equally from sites (Bancoumana 103 [52%; 95% CI 44·6–58·3]), and similar baseline parasitemia and gametocytemia rates (table 1).

Vaccinations in the low-dose, pilot-safety groups were well tolerated (appendix pp 23–25, 46–50).

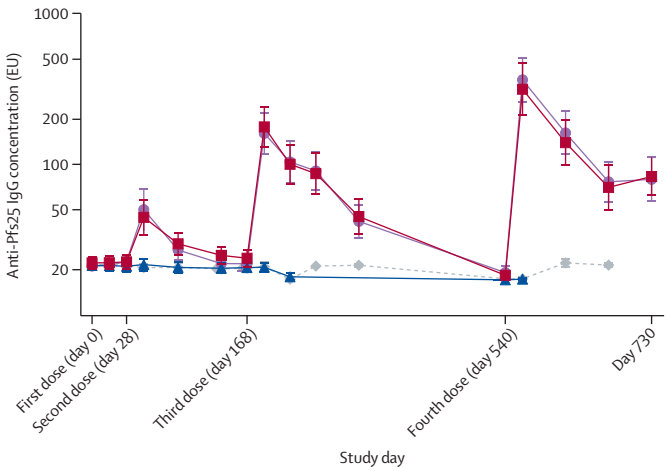
Vaccinations in the high-dose groups (47 µg Pfs25 and 40 µg Pfs230D1) were also well tolerated (table 2; appendix pp 25–30, 51–56). The most commonly reported AEs were grade 1–2 and most related AEs were injection site reactogenicity, reported more frequently in the Pfs25 and Pfs230D1 groups than in the comparator group (appendix pp 51–52). Reactogenicity was more common in the Pfs25 and Pfs230D1 limbs (and in comparator limbs after the second and fourth doses) than in saline limbs. Local reactogenicity in the Pfs25 plus Pfs230D1 group was attributed equally to either Pfs25 or Pfs230D1.

Solicited AEs were few (most commonly headache) and were only significantly more frequent (p=0·039) in the Pfs25 plus Pfs230D1 group after the fourth dose (appendix pp 53–54). Laboratory abnormalities were similar across groups and were grade 1–2 except for two unrelated grade 4 creatine increases (Pfs230D1 and Twinrix; appendix pp 55–56). Three unrelated SAEs (two in the Pfs230D1 group, snakebite and peritonsillar abscess; and one in the Pfs25 plus Pfs230D1 group, cerebrovascular accident resulting in death) are further detailed in the appendix (p 29). There were no grade 3–5 related AEs.

Malaria endpoints (symptomatic malaria, severity of malaria cases, parasitaemia, and gametocytaemia) were assessed throughout the study period. Comparison of malaria AE (frequency, duration, and severity), parasitaemia, and gametocytaemia showed no significant difference between vaccine versus comparator groups (table 2; appendix pp 29–30). Pilot cohort antibody titres against Pfs25 and Pfs230D1 are shown in the appendix (p 57).

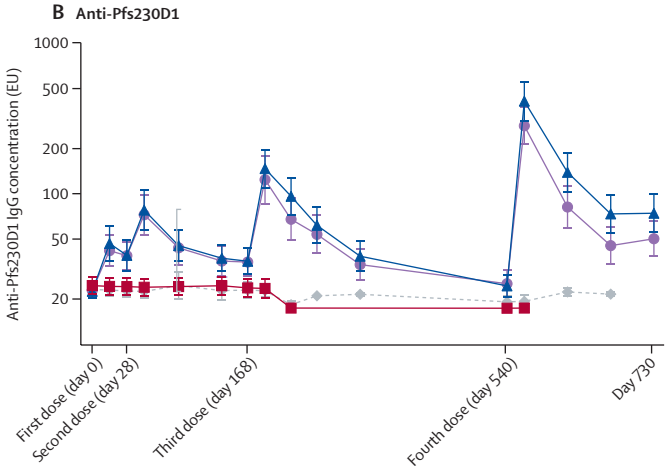
In the main cohort, antibody responses were first observed to be significantly higher than those in the comparator group 2 weeks after the second Pfs25 vaccination (p<0·0001 for Pfs25 alone; 44·7 EU [95% CI 34·0–58·7] and Pfs25 plus Pfs230D1 50·3 [36·5–69·3] *vs* comparator 20·8 [19·4–22·3]; figure 2A). By contrast, antibody responses were first observed to be significantly higher than those in the comparator after the first Pfs230D1 vaccination (p=0·0001 for Pfs230D1 alone; 46·7 [35·6–61·3] *vs* comparator, 22·9 [20·8–25·2]; p=0·0024 for Pfs25 plus Pfs230D1, 41·9 [32·6–53·7] *vs* comparator; figure 2B). Peak titres increased after each subsequent dose (Pfs230D1 geometric mean after the second dose 77·8 [95% CI 56·9–106·3], 146·4 [108·3–198·0] after the third dose, 410·2 [301·6–558·0] after the fourth dose; Pfs25 177·7 [130·3–242·4] after the third dose and 315·7 [209·9–474·6] after the fourth dose).

We also examined the number of doses needed to elicit an immune response, with seropositivity defined as the limit of detection (determined by the standard curve on each plate) averaged across all plates. Pfs230D1-alone responders were more frequent after one (20 [42%] of 48) or two doses (30 [71%] of 42) than Pfs25-alone responders



anti-Pfs25 ELISA units

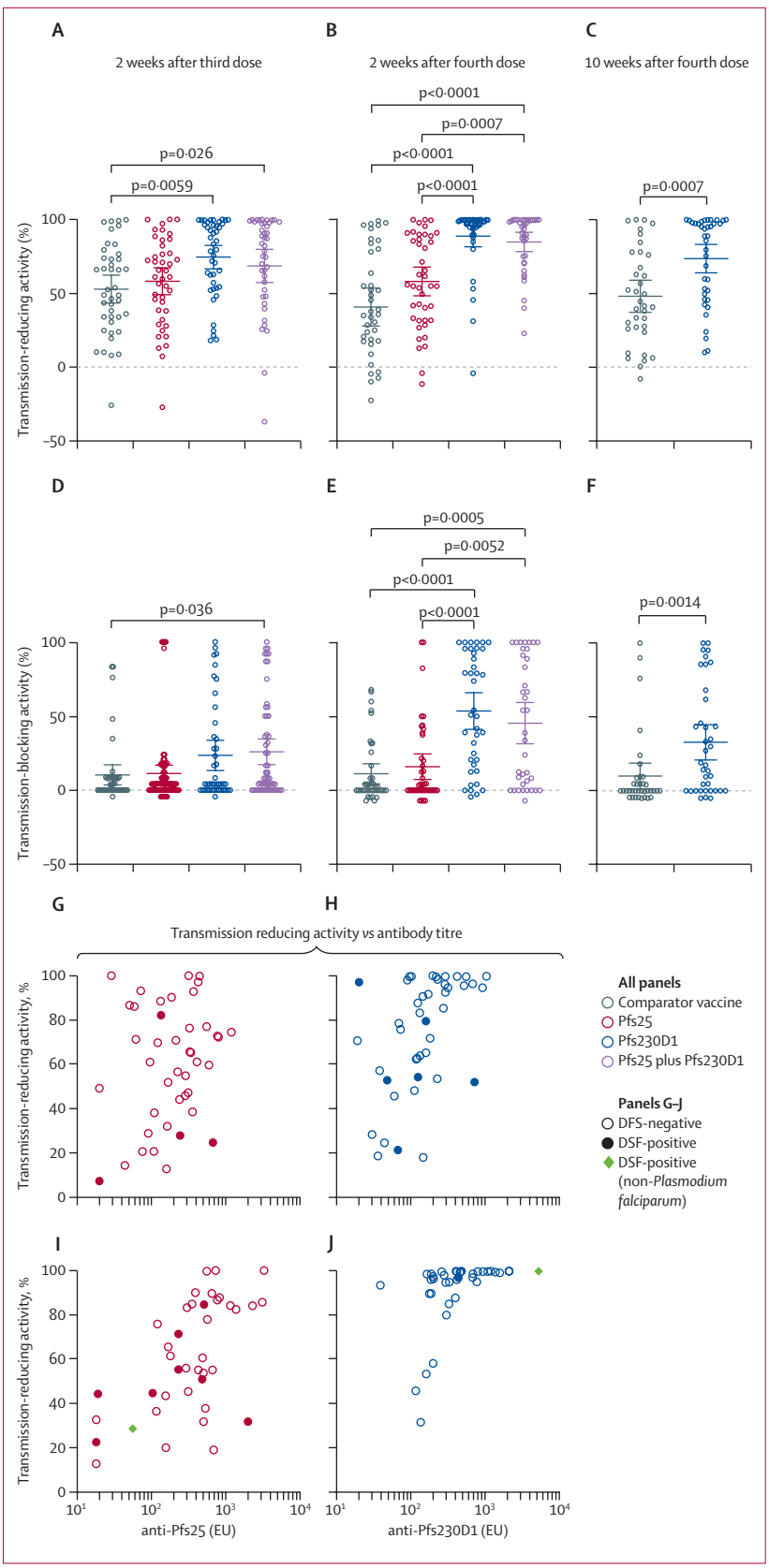
	Before first dose		After first dose		After second dose		After third dose		After fourth dose	
	Pfs25	Pfs25 plus Pfs230D1	Pfs25	Pfs25 plus Pfs230D1	Pfs25	Pfs25 plus Pfs230D1	Pfs25	Pfs25 plus Pfs230D1	Pfs25	Pfs25 plus Pfs230D1
Seropositive	2/50	0/50	2/48	1/48	20/45	19/48	37/43	36/43	38/42	36/37
Geomean	22·3	21·2	22·4	21·4	44·7	50·3	177·7	160·9	315·7	364·4
Median	21	20	21	19	38	32	214	133	412	321
Range	16–90	16–35	16–100	16–64	16–673	16–823	20–1194	20–1157	18–3322	24–3505
95% CI	20·1–24·6	19·8–22·8	19·9–23·4	19·6–23·4	34·0–58·7	36·5–69·3	130·3–242·4	115·7–223·8	209·9–474·6	256·2–518·2



anti-Pfs230D1 ELISA units

	Before first dose		After first dose		After second dose		After third dose		After fourth dose	
	Pfs25	Pfs25 plus Pfs230D1	Pfs25	Pfs25 plus Pfs230D1	Pfs25	Pfs25 plus Pfs230D1	Pfs25	Pfs25 plus Pfs230D1	Pfs25	Pfs25 plus Pfs230D1
Seropositive	2/49	3/50	20/48	22/48	30/42	31/48	38/43	34/43	38/39	37/37
Geomean	22·5	22·8	46·7	41·8	77·8	72·4	146·4	123·8	410·2	281·6
Median	21	20	30	33	64	53	146	115	399	300
Range	13–160	13–70	13–389	13–266	17–480	13–1091	19–1055	18–2022	39–5277	55–1759
95% CI	20·0–25·4	20·6–25·2	35·6–61·3	32·6–53·7	56·9–106·3	52·9–99·1	108·3–198·0	85·2–180·0	301·6–558·0	210·0–377·6

Figure 2: **Antibody titres for single and combination immunogen arms by ELISA**
Values are presented as EU. Geometric means are presented with error bars indicating 95% CIs. Differences in antibody titres induced by vaccines versus comparator were analysed by Mann-Whitney test. For anti-Pfs25 titres, significant differences were observed 2 weeks after the second, third, and fourth vaccinations ($p<0·0001$ for each Pfs25-containing group *vs* comparator). For anti-Pfs230D1 titres, significant differences were observed 2 weeks after the first vaccination ($p=0·0001$ for Pfs230D1 alone; $p=0·0024$ for Pfs25+Pfs230D1), and 2 weeks after each subsequent vaccination ($p<0·0001$ for each Pfs230D1-containing group). Comparator antibody titres to Pfs25 and Pfs230D1 were not completed for day 730. Anti-Pfs25 and anti-Pfs230D1 ELISA data at peak timepoints (2 weeks after vaccination) are provided in tables below each associated figure. Seropositive is defined as EU greater than mean + 3SD of plate level of detection (Pfs25=55 EU; Pfs230D1=43 EU). Post-vaccination receipt sample missingness (due to missed visit or off study post vaccination) was 0–3 participants per each timepoint and was evenly disbursed between groups (in the Pfs25 group, none before first dose, two off study after first dose, 3 missed visits after second dose, one off study after third dose, and none after fourth dose; in the Pfs230D1 group, none before first dose, one off study after first dose, 3 missed visits after second dose, none after third dose, and 1 missed visit after fourth dose; in the Pfs25 plus Pfs230D1 group, none before first dose, one off and one missed visit study after first dose; two off study after second dose, one off study and two missed visits after third dose, and one off study and one missed visit after fourth dose). EU=ELISA units.



after one (two [4%] of 48) or two doses (20 [44%] of 45; p<0·0001 after the first dose and p=0·017 after the second dose). Most vaccinees became seropositive after two Pfs230D1 or three Pfs25 doses and the proportion of responders after three and four doses was high and similar between groups (appendix pp 58–59). Seropositive individuals in the Pfs230D1 group were significantly more frequent in the Pfs230D1 alone versus combination groups at later timepoints after the fourth dose (at weeks 10 [p=0·031], 19 [p=0·0039], and 27 [p=0·060] after the fourth dose; appendix p 59), suggesting impaired antibody durability after co-administration.

Antibody responses remained higher than in the comparator group in both Pfs25-containing groups at 19 weeks after the third dose (p<0·0001 for Pfs25 alone, 45·3 EU [95% CI 34·5–59·6] and Pfs25 plus Pfs230D1, 42·0 [32·3–54·7] *vs* comparator 21·5 [20·8–22·2]; figure 2A), and at 19 weeks after the fourth dose (p<0·0001 for Pfs25 alone, 70·7 [49·4–101·0] and Pfs25 plus Pfs230D1, 76·9 [56·0–105·7] *vs* comparator, 21·6 [20·7–22·4]; figure 2A). Similarly, both Pfs230-containing groups showed significant antibody concentrations at 19 weeks after the third dose (p<0·0001 for Pfs230D1 alone 38·5 [30·5–48·6] and Pfs25 plus Pfs230D1, 33·8 [26·6–43·0] *vs* comparator, 21·5 [20·8–22·2]; figure 2B), and at 19 weeks after the fourth dose (p<0·0001 for Pfs230D1 alone, 73·3 [54·5–98·5] and Pfs25 plus Pfs230D1, 45·1 [33·8–60·3] *vs* comparator, 21·5 [20·8–22·4]); figure 2B).

At the time of the fourth vaccination dose, anti-Pfs25 antibody concentrations were not significantly different between Pfs25-containing groups and comparator groups, whereas anti-Pfs230D1 concentrations remained significantly higher in the combination group (but not Pfs230D1 alone) versus comparator (p=0·017 for combination group and p=0·15 for single Pfs230D1 antigen; figure 2B). Individuals with pre-existing titres to Pfs25 or Pfs230 as measured at baseline visit did not have significantly different titers from participants in the same group.

***Figure 3*: Transmission-reducing activity, transmission-blocking activity, and durability**

Transmission-blocking vaccine functional activity was assessed by standard membrane feeding assay. For 2 weeks after each vaccination (after third dose [A, D]; after fourth dose [B, E]), differences between groups were analysed by Kruskal-Wallis test with Dunn's correction for multiple comparisons; at 10 weeks after dose 4 (C, F), differences between Pfs230D1 group and comparator were analysed by Mann-Whitney test. Significant p values are presented. Transmission reducing plotted as a function of ELISA titre for Pfs25 and Pfs230D1 single-antigen groups at 2 weeks after third dose (G, H), and 2 weeks after fourth dose (I, J). Results for the Pfs25 plus Pfs230D1 combination group are presented in the appendix (p 67). Results for transmission reducing activity at 10 weeks after the fourth dose for Pfs230D1 single-antigen group are in the appendix (p 68). Empty circles represent participants with negative DSF results, closed circles indicate participants with positive DSF results, green diamonds indicate DSFs that were positive for a non-falciparum *Plasmodium* species detected by PCR analysis of a single midgut selected from the feed. Dotted horizontal lines represent no difference from assay control (non-immune sera). DSF=direct skin feed.

In exploratory analysis, we compared ELISA responses in this Malian population to a US safety cohort (n=5 per group) who received two vaccine doses under this protocol as previously reported.[11] Pfs25 antibody concentrations were higher in US than Malian participants in response to high-dose (47 µg) Pfs25 at 2 weeks (day 42, p=0·0086) and 8 weeks (day 84, p=0·031) after the second dose (appendix p 60). By contrast, Pfs25 antibody concentrations induced by the low dose (16 µg) Pfs25 single antigen might be higher in Malian than US participants at 2 weeks (day 42, p=0·067). Pfs230D1 antibody concentrations did not significantly differ between US and Malian populations (appendix p 60).

Responses to EPA appeared after the first dose in all vaccine groups and geometric mean titres increased with successive doses (appendix pp 31–32, 61). Post-vaccination EPA titres were consistently higher in the combination group versus single-antigen groups (appendix pp 31–32, 61).

Stool co-infections reduced Pfs230D1 titres after the second dose, but not after the third (appendix p 33).

Proportions of responders at peak titres (2 weeks after vaccination) did not significantly differ in supplementary modeling analyses (appendix p 62). Supplementary Bayesian models for immune responses suggested that the combined administration of Pfs230D1 and Pfs25 did not affect the number of doses needed to elicit a Pfs230D1 immune response compared with administering Pfs230D1 alone (common odds ratio 1·08 [95% CI 0·52–2·21]) or Pfs25-alone (0·86 [0·4–1·82]). Posterior log geometric mean titres were estimated for Pfs230D1 and Pfs25 (appendix p 63): although log geometric mean titres only slightly exceeded limit of detection after the second Pfs25 dose, they already exceeded assay limit of detection after the first Pfs230D1 dose.

Supplementary Bayesian antibody decay models suggested Pfs230D1 antibodies were more durable than Pfs25 antibodies (appendix p 62–66). We estimated that after 16 weeks, Pfs230D1 geometric mean titres were 30% (95% CI 26–34) of peak versus 22% (19–26) for Pfs25 (appendix p 62–66). Durability of the response was not greater in the combination group versus single antigen vaccination groups: estimated Pfs230D1 titres were slightly higher in the Pfs230D1-alone group versus combination group following the third and fourth doses, whereas Pfs25 titres did not differ between single and combination groups (appendix p 66).

Serum functional activity was assessed by SMFA in each group using all available sera from the as-treated population collected 2 weeks after the third and fourth doses, and in the Pfs230D1 monovaccination and comparator vaccine groups at 10 weeks after the fourth dose. Significant transmission-reducing activity appeared for Pfs230D1 and Pfs25 plus Pfs230D1 after the third dose (mean 74·5% [95% CI 66·6–82·5] and 68·6% [57·3–79·8]) and after the fourth dose (88·9% [81·7–96·2] and 85·0% [78·4–91·5]) but not for Pfs25 (58·2% [49·1–67·3] and 58·2 [48·5–67·9]) after the third and fourth doses (figure 3A, B, D, E). Pfs230D1 transmission-reducing activity (73·7% [64·1–83·3%]) persisted 10 weeks after the fourth dose (figure 3C, F).

To relate titres and functional activity (transmission-reducing activity), linear regressions were fitted regressing transmission-reducing activity onto the $\log_{10}$ ELISA titres at 2 weeks after the third dose (figure 3G, H) and 2 weeks after the fourth dose (figure 3I, J) for single antigens; the effect was significant in each model. To achieve a transmission-reducing activity of 80%, we estimated that an ELISA value of 1659 would be required for the Pfs25 vaccine, compared with an ELISA value of 218 for the Pfs230D1 vaccine (figure 3G–J). For the Pfs25 plus Pfs230D1 combination vaccine, transmission-reducing activity of 80% required a similar Pfs230D1 ELISA value of 223 (appendix p 67). The relationship for the Pfs230D1-alone group appeared similar 10 weeks after vaccination four (appendix p 68).

DSF assays were done twice per week for 6 weeks after the third and fourth doses, using 60 (year 1) or 30 (year 2) mosquitoes per feed (evident as two distributions of mosquito numbers dissected among the negative DSFs; appendix p 69). After 3850 DSF assays, 35 participants (eight [23%] Pfs230D1, 12 [34%] Pfs25, five [14%] Pfs25 plus Pfs230D1, and 10 [29%] comparator) had transmitted parasites at least once (figure 4; appendix p 70). As expected,[1] a small proportion of DSFs were positive, typically with a minority of mosquitoes infected (figure 4; appendix p 69).

The proportion of positive assays in vaccine groups (Pfs25 33 [3%] of 982, Pfs230D1 22 [2%] of 954, and combination 11 [1%] of 940; figure 4; appendix p 70) did not differ from that of the comparator (22 [2%] of 974), nor did Pfs230D1 and combination groups differ (figure 4; appendix p 70). The low rate of DSF positivity limited power to detect differences.

Although only 35 (20%) of the 175 participants who underwent at least one DSF assay had a positive DSF during follow-up, DSF-positive participants were as likely to transmit parasites in two or more assays (12 [67%] of 18 DSF-positive individuals in season 1 and 9 [50%] of 18 in season 2) as to transmit during a single assay during the same season (figure 4; appendix pp 70–75). Multiple positive feeds occurred consecutively in several individuals. Conversely, only one participant had positive DSF in both seasons, which is no greater than that predicted by random chance (enrichment analysis predicted 2·1 individuals with positive DSF both seasons; p=0·36).

We assessed DSF oocyst counts and DSF-infected mosquitoes in relation to ELISA antibody responses in zero-inflated negative binomial random effect models (appendix pp 76–77). No significant associations were found in these models that explored peak dose-4 ELISA titre relationships to DSF outcomes (appendix pp 76–77).

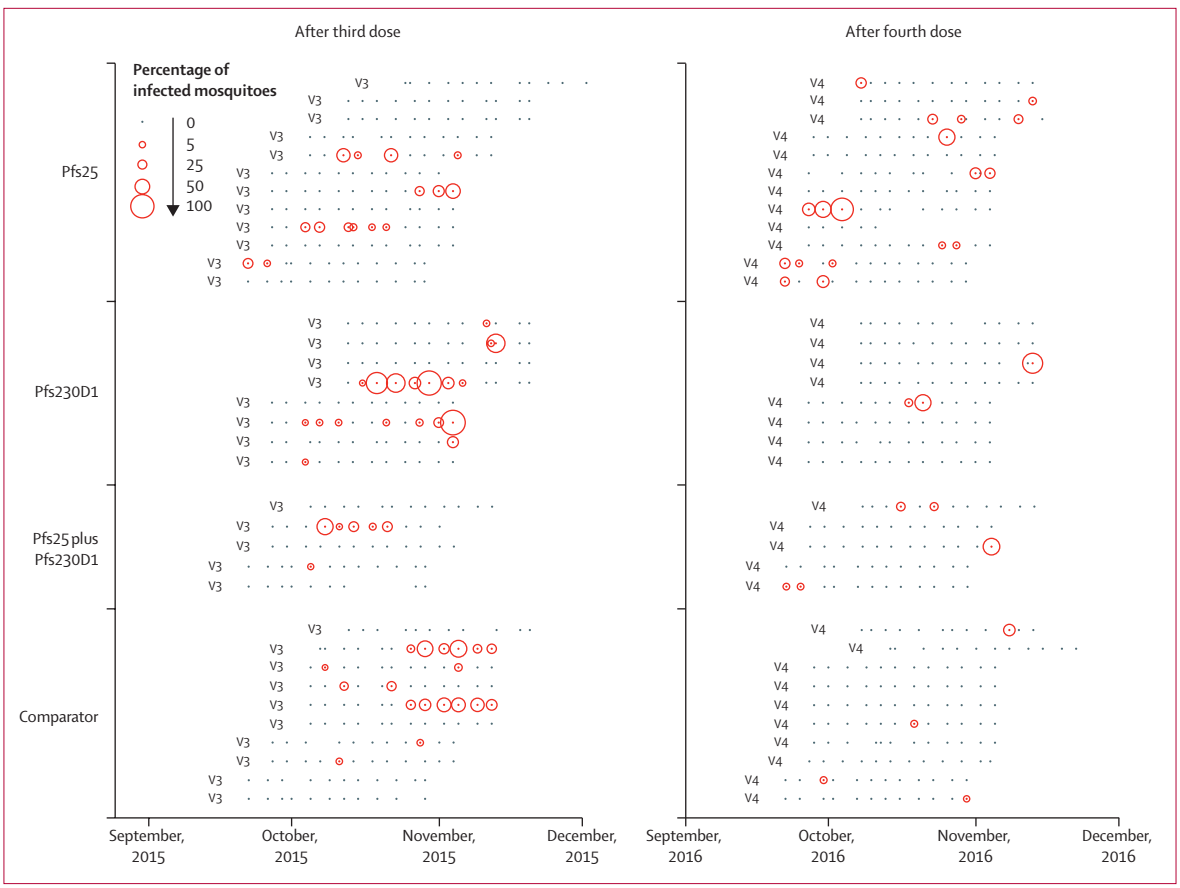


Figure 4: **DSF assay results for participants who transmitted parasites in at least one DSF assay (n=35)**
Of 175 individuals who underwent at least one DSF assay, 35 transmitted parasites on at least one occasion. One of these 35 DSF-positive participants yielded positive transmission events in both years, 17 in year 1 only, and 17 in year 2 only. Each participant is depicted by timelines over two seasons that indicate DSF timepoints and their outcomes, stratified by trial group. Feeds used 60 mosquitoes in year 1 and 30 in year 2. Dissections were done 1 week after feed to assess transmission, with each surviving mosquito surveyed for the presence of oocysts with feeding and survival rates per arm available in the appendix (p 70). The dots and colored circles in the figure represent DSF assays, with small black dots denoting negative DSF where no dissected mosquitoes had oocysts. Red circles are positive DSF transmission events, with the circle size proportional to the percentage of dissected mosquitoes that contained oocysts. DSF=direct skin feed.

Forensic typing of blood-fed mosquitoes collected from the experimental hut suggested a high rate of contamination by mosquitoes (66 [35%] of 189) that had fed outside of the hut (appendix pp 32–33), limiting the usefulness of the approach to assess vaccine effectiveness.

Discussion

Gains in malaria control have plateaued since 2015 and reversed in high-burden regions,[2] mandating new control tools. Here, we report two leading transmission-blocking vaccine candidates to be safe, alone or in combination, in adults in rural Mali. Seroconversion occurred after fewer Pfs230D1 than Pfs25 doses, and Pfs230D1 antibodies persisted longer. Pfs230D1 serum functional activity appeared after the third dose and persisted at least 10 weeks after the fourth dose; Pfs25 did not induce significant functional activity and did not enhance Pfs230D1 activity in a combination vaccine. These data firmly establish Pfs230D1 superiority over Pfs25 in the target population.

We earlier reported that Pfs230D1 induces functional activity after two doses in malaria-naive US participants,[11] whereas Pfs25 in Alhydrogel required four doses to achieve significant activity in most US[10] or Malian vaccinees.[1] Here, Pfs230D1 but not Pfs25 conferred statistically significant functional activity after both primary vaccine series and booster dose that persisted at least 10 weeks after dose four. Using human monoclonal antibodies generated from this trial, we previously observed that Pfs230D1 functional antibodies bind native antigen on the gamete surface, fix complement and lyse the parasite; non-functional Pfs230D1 antibodies often fail to bind native antigen, probably because epitopes are masked by downstream Pfs230 domains.[1,10]

As in US adults,[11] the Pfs230D1 plus Pfs25 combination in Malian adults did not enhance immunogenicity over single antigens nor improve serum functional activity achieved with Pfs230D1 alone. Peak antibody concentrations against Pfs230D1 (but not Pfs25) were lower with the combination, although differences were not

statistically significant. At later post-dose four timepoints, the proportion of Pfs230D1-seropositive individuals was significantly greater in the Pfs230D1 alone versus combination group. Future studies should examine the combination at different doses or using different adjuvants.

Pfs230 but not Pfs25 is expressed during gametocyte development in humans, and exposed populations acquire Pfs230 antibodies. Naturally acquired transmission-reducing activity has been related to Pfs230 antibodies,[18] and Pfs230 vaccines hypothetically might benefit from antibody boosting during *P falciparum* infections. Conversely, pre-existing antibody responses can impair vaccine responses, as suggested for the malaria circumsporozoite protein repeat-region.[19] Here, Pfs230D1 responses did not significantly differ between Malian and US populations, whereas Pfs25 responses after the first and second doses were significantly lower in Mali, similar to our previous study.[1] Pfs230D1 responses did not differ between participants by pre-existing antibody status (appendix p 78). Naturally acquired transmission-reducing activity might have hindered our ability to statistically confirm the Pfs25 functional activity that appeared to increase after the fourth vaccine dose.

As transmission-blocking vaccine clinical development advances, efficacy trials will require large cluster-randomised designs to measure herd immunity.[19] We are exploring DSF as an interim endpoint, whereby colony-raised mosquitoes are fed directly on trial participants.[20] Here, we established safety and tolerability of twice per week DSF, which yielded measurable endpoints (infected mosquitoes) but with too few unique DSF-positive individuals to confirm vaccine effectiveness. DSFs with 30 mosquitoes detected fewer transmission events versus DSFs with 60 mosquitoes. Conversely, transmission events were often observed in consecutive DSF, suggesting oversampling with our twice-weekly schedule. In future, we plan to reduce DSF sampling frequency and extend the period of DSF using 60 mosquitoes per feed to achieve sufficient endpoint events in unique individuals and confirm vaccine effectiveness in vivo.

Overall, our results are promising. Pfs230D1 requires fewer doses than Pfs25 for serum functional activity, Pfs230D1 antibodies persist longer, and Pfs230D1 serum activity persists at least 10 weeks after the fourth dose, unlike Pfs25 activity that disappeared within 8 weeks in previous testing.[10] In preclinical studies, more potent adjuvants such as liposomal formulations[21] improved durability of Pfs25 and Pfs230D1 vaccines.[22–24] We are now investigating Pfs230D1-EPA and Pfs25-EPA with such adjuvants in humans (NCT02942277, NCT03917654, NCT05135273, and ISRCTN13649456).

Our study had limitations. Dropout rates were similar in study groups over the 2-year trial, but selection bias might have occurred because of missing outcome data. Our DSF assays that measured the rate at which participants transmit naturally circulating parasites to mosquitoes detected transmission events in a low proportion of participants. Future trials should perform direct skin feed assays less frequently over a longer time period to increase the proportion of participants with detectable transmission. Trials in younger age groups known to transmit parasites more frequently[25] might increase power to measure vaccine effectiveness.

In conclusion, Pfs25 and Pfs230D1 conjugate vaccines adjuvanted with Alhydrogel are safe, well tolerated, and immunogenic in Malian adults. Pfs230D1 is superior to Pfs25 on the basis of functional immunogenicity by SMFA and combining Pfs230D1 with Pfs25 does not improve serum functional activity. DSF is a valuable in-vivo measurement of parasite transmission but must be further optimised to enhance statistical power for vaccine trial endpoints.

Contributors

SAH, OKD, and PED conceived the study. IS and SAH were the trial principal investigators. IS, SAH, JCCH, YW, MBC, SFT, OKD, and PED designed the trial. IS, MHA, MK, KS, BK, YS, MAG, MD and DS collected the data. MAG, MD, IZ, FS-M, JD, JN, OM, DS, EMO, JCCH, and CFA completed the study laboratory endpoints. KMR, DLN, DLJ, NJM, DZ, and PED developed the vaccine. RM and IT developed and managed the database for the study. AD and KN prepared the vaccines for injection. BJS, JLK, JF, and RDM completed the statistical analysis and modeling. IS, SAH, BJS, JLK, BD, JPG, AI, RM, RDM, and JCCH curated the data for interpretation and manuscript preparation. IS, SAH, BJS, IZ, RDM, YW, JCCH, MBC, CFA, OKD, and PED interpreted data and results. Clinical principal investigators (IS, SAH) had full access to all study data and responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

All data associated with this study are present in the paper or appendix and are available from the authors upon reasonable request. Individual-level deidentified participant data will be made available with publication and upon execution of inter-institutional human data sharing agreement. Data can include all those described in the manuscript.

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